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Attorney's Docket No.: 14184-008001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Robert W. Busby et al. Art Unit : 1636
Serial No. : 09/487,558 Examiner : K. Davis
Filed : January 19, 2000
Title : METHODS FOR IMPROVING SECONDARY METABOLITE PRODUCTION IN FUNGI

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUBMISSION OF SIGNED DOCUMENT

Enclosed is the signed Declaration of Edward Driggers Under 37 C.F.R. §1.132. An unsigned Declaration was submitted (as Exhibit A) with the Reply to Office Action filed on January 15, 2004.

Please apply any charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 4 Feb 2004

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CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

February 4, 2004
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Carrie A. Amonte
Signature

Carrie A. Amonte
Typed or Printed Name of Person Signing Certificate

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Applicant : Robert W. Busby et al. Art Unit : 1636
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DECLARATION OF EDWARD DRIGGERS UNDER 37 C.F.R. §1.132

1. I, Edward Driggers, am a Senior Scientist I at Microbia, Inc. I received a Ph.D. in Organic Chemistry from the University of California, Berkeley in 1998 and an S.B in Biological Chemistry from the University of Chicago in 1989. I was a National Science Foundation-Sloan Fellow in Molecular Evolution at Stanford University from 1998 to 2001.

2. I and others working at Microbia, Inc. conducted experiments to investigate the effect of CreA expression on the production of certain polyketides produced by *A. terreus* and a polyketide produced by *P. citrinum*.

Effect of CreA on production of polyketides in *A. terreus*

3. In order to investigate the effect of CreA expression on the production of polyketides produced by *A. terreus*, a vector (MB3131) expressing *A. nidulans* CreA

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under the control of the *A. nidulans* PGK promoter, was transformed into *A. terreus* (strain ATCC 20542) using standard protocols. The production of various polyketides in CreA transformed cells was then compared to their production in non-transformed *A. terreus* control cells.

Transformation of *A. terreus* with a CreA expressing vector

4. *A. terreus* were transformed by generating protoplasts from spores that were germinated in rich media (2% yeast extract, 6% sucrose, 0.9M sorbitol). Spores were allowed to germinate for about 20 hrs or until germ tubes were between 5 and 10 spore lengths. The germlings were centrifuged and washed twice with sterile distilled water and once with 1M magnesium sulfate. Germlings were then resuspended in 1M magnesium sulfate containing approximately 2mg/ml of Novozyme and incubated at 30°C shaking at 80 rpm for about 2 hours or until most of the hyphae were digested and protoplasts were abundant. Protoplasts were then filtered through one layer of Miracloth. At least one volume of STC (0.8M Sorbitol, 25mM Tris-HCl pH 7.5, 25mM $CaCl_2$) was added and protoplasts were centrifuged and then washed twice with STC. Protoplasts were then resuspended in 1ml STC and counted in a hemocytometer. Protoplasts (approximately 5 x 10^7 protoplasts/ml) were frozen in a 9:1:0.1 solution of STC, SPTC and DMSO in a Nalgene Cryo cooler at -80°C (cools -1°C/min). For transformation, 1-5 μg of vector DNA was placed in a 50 ml Falcon tube along with 100 μl of previously frozen protoplasts. The transformation mixture was gently mixed and then incubated on ice for 30 minutes. Next, 15 μl of SPTC (0.8M Sorbitol, 40% PEG 4000, 25mM Tris-HCl pH 8, 50mM $CaCl_2$) was added, followed by mixing and incubation at RT for 15 minutes. 500 μl SPTC was then added and mixed well by rolling and this mixture was incubated at RT for 15 minutes. Next, 25 mls of regeneration minimal medium (trace elements, salts, 25mM sodium nitrate, 0.8M sucrose, and 1% agarose at pH 6.5) was added, mixed well and poured on plates containing 25 mls of regeneration minimal medium with 2X the concentration of selection drug. Transformation plates were incubated at 28°C for 5-6 days or until colonies started to appear. Transformants were picked onto new plates with a toothpick (if fungus was sporulating) or with sterile forceps (if fungus did not sporulate). Purification plates contained minimal medium (same as regeneration minimal medium but containing 2% instead of 0.8M sucrose) and 1X antibioitic drug concentration (for *A. terreus*). Picked transformants were incubated at 28°C for 5-6 days before shake flask inoculation.

Measurement of polyketide production

5. Transformants were grown in production media to assess polyketide production. The production media was essentially that described in U.S. Patent 6,391,583. Spores obtained from the purification plate using a wooden inoculation stick were used as the inoculum. The medium was RPM containing corn steep liquor, sodium nitrate, potassium phosphate, magnesium sulfate, sodium chloride, P2000 (Dow Chemical), trace elements and lactose or glucose as carbon source. The medium was buffered to pH 6.5. Flasks were incubated at 28°C with shaking at 225 rpm.

6. To measure the production of various polyketides in *A. terreus*, a sample of broth (200 μL) from a 5-day shake flask culture was filtered (0.45 um) and diluted 1:5 into aqueous 30% acetonitrile. Metabolites were resolved and identified using a Micromass quadropole time-of-flight (Q-ToF) mass spectrometer connected to a Waters 2795 HPLC system. Samples were injected (20 μL) and resolved using reverse phase HPLC (a linear gradient from 65-90% of acetonitrile containing 0.1% formic acid in aqueous 0.1% formic acid eluted the samples from a Waters Xterra MC-C18 (2.1 x 150 mm, 2.5 um particles) over the course of 22 minutes at a flow rate of 0.2 mL/min.). The Q-ToF mass spectrometer was set to detect masses between 50 and 950 Da, and was calibrated by infusion of a poly-alanine standard immediately prior to operation. A mass reference was co-infused post-column to allow high resolution mass data to be acquired. Identified masses were compared with a high-resolution mass database of known *A. terreus* metabolites. The relative abundance of metabolites was determined by integration of peak areas from an extracted mass chromatogram within a mass window of 10 ppm of the expected mass for a given metabolite.

7. The production of the various polyketides by *A. terreus* that were not transformed with the CreA expressing vector was measured in an identical manner and compared to production by the CreA transformed cells in order to determine whether CreA expression altered production of the various polyketides. Briefly, three CreA transformed clones were selected. Three cultures were then grown from each of these

clones and assayed. The same was done with non-transformed *A. terreus*. Thus, for each polyketide there are twelve total measurements, three each from the three CreA transformed strains, and three measurements in non-transformed cells. The results of this analysis are presented in the table below where the polyketide production in CreA transformed cells relative to non-transformed cells is reported along with the p-value from the statistical analysis (a two-sided t-test assuming unequal variances). The structures of the polyketides and their location in the geodin or lovastatin biosynthetic pathway are depicted in Exhibit A (attached). In each case, the polyketide structure is boxed and identified by both name a reference number from the table.

Polyketide	**Polyketide production ratio in CreA transformed cells relative to non-transformed cells** **P value**	**Is this polyketide part of a statin pathway?**	**CAS registry number**	**Reference number**
3,5-Dihydro-3-hydroxymonacolin L	6.8 ($p=7.7 \times 10^{-10}$)	yes	119786-66-2	1
Acetyl Lovastatin, closed	7.6 ($p=7.3 \times 10^{-7}$)	yes	Not available	2
Erdin; (%)-form 7-Dechloro, Me ester	0.46 ($p=0.0137$)	no	103470-59-3	3
Erdin; (R)-form (lacking the methylester on the bottom)	4.2 ($p=9.73 \times 10^{-5}$)	no	26891-81-6	4
Mevinolin; 4,4a-Dihydro	5.5 ($p=7.99 \times 10^{-8}$)	yes	77517-29-4	5
Monacolin J; 1-Deoxy	7.2 ($p=1.29 \times 10^{-9}$)	yes	79394-47-1	6
Osoic acid; 3-Me ether, 1-Me ester	0.48 ($p=0.00334$)	no	577-64-0	7
Tiglistatin, open	>10*	yes	Not available	8

*The production of tiglistatin by non-transformed cells was too low to be detected, but based on the apparent lower limit of detection in this assay it was estimated that CreA transformed cells express at least 10-fold more tiglistatin than non-transformed cells.

8. As can be seen from the table, transformation with CreA increased the production of six different polyketides and reduced the production of two. The results of this study demonstrate that increased expression of CreA modulates polykektide production in *A. terreus*.

9. Askenazi et al. (2003 *Nature Biotechnology* 21:150), of which I am a co-author, discloses the results of studies showing that transformation of *A. terreus* with a vector expression CreA increased geodin production 2.6-fold and lovastatin production 4.16-fold compared to control A. terreus transformed with an empty vector not expressing CreA.

Effect of CreA on production of compactin in *P. citrinum*

10. In order to investigate the effect of CreA expression on the production of compactin, a polykektide produced by *Penicillium citrinum*, a vector (MB3405) expressing *A. nidulans* CreA under the control of the *A. nidulans* PGK promoter, was transformed into *P. citrinum* (strain ATCC 20606) using standard protocols. The production of various polyketides in CreA transformed cells was then compared to *P. citrinum* cells transformed with an empty vector (no CreA sequence).

Transformation of *P. citrinum* with a CreA expressing vector

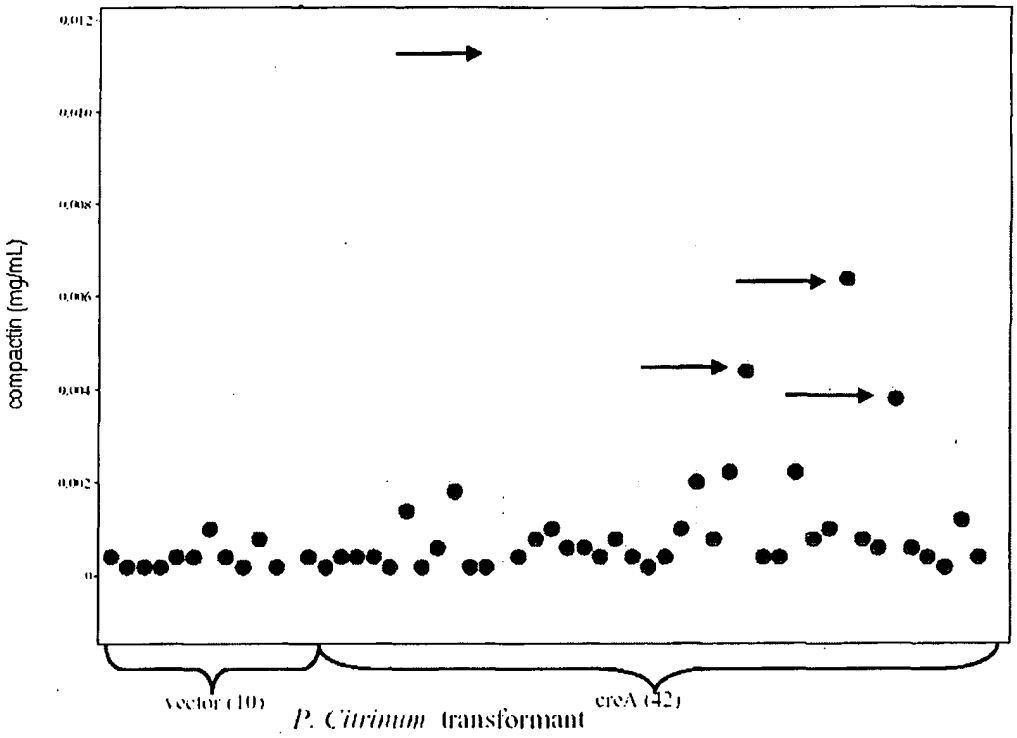
11. Transformation of *P. citrinum* was performed essentially as described above for *A. terreus*.

Measurement of compactin production

12. Following regeneration, transformants were transferred to minimal medium with 250 μg/ml hygromycin and grown at 28°C for 7 days before shake flask inoculation. Biomass was transferred, using sterile wooden sticks, from 12 well plates to 125 ml shake flasks containing 20 ml of MBG3-8 media (7% glycerin, 3% glucose, 1% soybean powder, 1% peptone, 1% corn steep liquor, 0.5% sodium nitrate, 0.1% magnesium sulfate heptahydrate, (pH6.5). These culture conditions are essentially those described by Hosobuchi et al. (1993) *Bioscience, Biotechnology, and Biochemistry* (1993), 57(9):1414-19). Cultures were grown for 12 days (24°C, 250 RPM). To

measure compactin, cultures of *P. citrinum* were extracted whole by shaking for 1 hour with an equal volume of ethanol (EtOH) containing 0.1 M NaOH. The extract was filtered, and the filtrate diluted 1:5 into aqueous 0.1% formic acid. These samples were analyzed by LCMS for compactin concentration. Samples were injected (10 μL) onto a reverse phase HPLC column (Betasil C18, 2.1 x 50 mm, 3 μm particle size) at 23°C, and eluted under isocratic conditions (40% aq. 0.1% formic acid, 60% acetonitrile containing 0.1% formic acid) for 4 minutes. Compactin eluted from the column at 1.9 minutes, and was detected by triple-quadrapole mass spectrometry (MRM, 431>329.1 Da; cone voltage = 20 V; collision = 26 eV). Instrument response was converted into concentration units by comparison with a standard curve using known amounts of authentic compactin.

13. The figure below shows the compactin levels for 10 vector only transformed clones and 42 creA transformed clones. A 2-sided T-test assuming unequal variances was performed. When all data points were included, p=.012; when the 4 highest creA data points were excluded (indicated by arrows), p=.019. Thus, in both cases, p<.05 demonstrating that the differences seen in the two populations is highly significant and thus creA increases compactin levels in *P. citrinum.*



14. I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant patent application or any patent issued thereon.

Date: 2/2/04

Edward Driggers, Ph.D.